A novel ribonuclease with antiproliferative activity toward leukemia and lymphoma cells and HIV-1 reverse transcriptase inhibitory activity from the mushroom, *Hohenbuehelia serotina*

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Abstract. In this study, a 27-kDa ribonuclease (RNase) was purified from the dried fruiting bodies of the mushroom, *Hohenbuehelia serotina*. The isolation protocol involved anion exchange chromatography, affinity chromatography, cation exchange chromatography and gel filtration in succession. The RNase was unadsorbed on DEAE-cellulose, but was adsorbed on Affi-gel blue gel and CM-cellulose. The N-terminal amino acid sequence was TVGGSLAEKGN which showed homology to other fungal RNases to a certain degree. The RNase exhibited maximal RNase activity at pH 5 and 80˚C. It demonstrated the highest ribonucleolytic activity toward poly(C), a relatively high activity toward poly(U), and a considerably weaker activity toward poly(A) and (G). The RNase inhibited human immunodeficiency virus type 1 (HIV-1) reverse transcriptase with an IC_{50} of 50 μM and reduced [^{3}H-methyl]-thymidine uptake by L1210 leukemia cells and MBL2 lymphoma cells with an IC_{50} of 25 μM and 40 μM, respectively.

Introduction

The literature pertaining to the *Hohenbuehelia* species is limited and confined to the following reports. Vafina and Molodtsov described the synthesis of some p-nitrophenyl 2-acylamino-2-deoxy-D-glucosides and their hydrolysis using *Hohenbuehelia serotina* β-D-hexosaminidase (1). The culture filtrate of *Hohenbuehelia geogenius* inhibited the growth of two rapidly growing grafted tumors (Ehrlich ascites carcinoma and L1210 lymphoid leukemia) and a slow-growing spontaneous mammary tumor in mice. An active substance was isolated by solid-liquid extraction and column chromatography and its chemical structure was elucidated (2).

Anti-A agglutinins, the reaction of which has been shown to be strongly inhibited by N-acetyl-D-galactosamine, have been detected in *Hohenbuehelia serotina* extracts (3). Two strains of *Hohenbuehelia atrocaerulea* (Pleurotaceae) that produce pleurotin, a naphthoquinone antibiotic originally obtained from *Pleurotus griseus*, have been identified. Solid substrate fermentation for two months yielded 1-2 mg/l of pleurotin. Shipley *et al* (4) detailed the developmental process, which depended on inclusion in the medium of an aqueous extract of alder woodm leading to a yield of pleurotin exceeding 300 mg/l from liquid fermentation. Bala *et al* reported that water, ethanol and hexane extracts of the *Hohenbuehelia* species inhibited growth of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* (5). Bala *et al* (6) further demonstrated that a water extract of *Hohenbuehelia* species inhibited six pathogens each comprising of two Gram-positive and -negative bacteria together with two fungi.

Polysaccharides extracted from the fruiting bodies of *Hohenbuehelia serotina* have exhibited antitumor activity in sarcoma 180-bearing mice (7). The molecular weights of these polysaccharides ranged from 1.19x103 to 1.55x104 Da and composed of ribose, arabinose, mannose, glucose and galactose at a ratio of 0.65:0.69:9.35:14.24:5.47; they were isolated by Li *et al* (8). However, there is a dearth of information on the proteinaceous constituents of the *Hohenbuehelia* species.

Ribonucleases (RNases) have been isolated and characterized from a multitude of organisms, including parasites, bacteria, fungi, plants and a variety of tissues from mammals (9-17) RNases display different activities, such as antitumor (18-25), immunosuppressive (26), antifungal (27), and antiviral (28,29) activities. Due to the array of potentially exploitable activities, RNases have drawn the attention of many researchers.

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RNases have been purified from the fruiting bodies or mycelia of a diversity of mushroom species. These mushrooms include some common edible and medicinal species as follows: *Pleurotus ostreatus* (30,31), *Irpex lacteus* (32), *Volvariella volvacea* (33), *Pleurotus tuber-regium* (34), *Pleurotus pulmonarius* (35), *Agrocybe cylindracea* (36), *Russula virescens* (37), *Termitomyces globules* (38), *Cantharellus cibarius* (39), *Pleurotus sajor-caju* (40), *Ganoderma lucidum* (41), *Clitocybe maxima* (42), *Thelephora ganbajun* (43), *Boletus griseus* (44), *Hypsizigus marmoreus* (45), *Russula delica* (16,46) and *Lyophyllum shimeiji* (47). The aim of this study was to isolate and characterize an RNase isolated from the dried fruiting bodies of the edible fungus, *Hohenbuehelia serotina*, and to compare its characteristics and N-terminal sequence with those of RNases isolated from the aforementioned species. The comparison would reveal any differences between RNases from different species and our findings may expand the knowledge and provide further information on this fungus.

Materials and methods

Isolation of RNase from Hohenbuehelia serotina. The dried fruiting bodies (200 g) of the edible mushroom, *Hohenbuehelia serotina,* obtained from North China were extracted with distilled water (2 ml/g) using a Waring blender (Waring Laboratory Supplies, Torrington, CT, USA). Tris-HCl buffer (pH 7.2, 1 M) was added to the supernatant, obtained by centrifugation of the homogenate, until the concentration of Tris reached 10 mM. The supernatant was subjected to ion exchange chromatography on a 5x15 cm column of DEAE-cellulose (Sigma, St. Louis, MO, USA) in 10 mM Tris-HCl buffer (pH 7.2). Following elution of the unadsorbed proteins (fraction D1) with the same buffer, the adsorbed proteins were desorbed sequentially with 0.2 M NaCl and 1 M NaCl in Tris-HCl buffer to form fractions D2 and D3, respectively. Fraction D1 was then directly chromatographed on a 5x15 cm Affi-gel blue gel column (Bio-Rad, Hercules, CA, USA) in 10 mM Tris-HCl buffer (pH 7.2). The unadsorbed proteins were eluted as fraction B1. The adsorbed proteins were eluted sequentially with 0.2 M NaCl and 1 M NaCl in Tris-HCl buffer and collected as fractions B2 and B3, respectively. Fraction B2 was dialyzed against 10 mM NH_4OAc buffer (pH 5) subjected to ion exchange chromatography on a 2.5x20 cm CM-cellulose (Sigma) column in 10 mM NH_4OAc buffer (pH 5). Following the removal of the unadsorbed proteins (fraction CM1), the adsorbed proteins were eluted with 10 mM NH_4OAc buffer (pH 5) containing a linear gradient of 0-1 M NaCl, and collected as fractions CM2 and CM3. Fraction CM3 was dialyzed, lyophilized and further purified on a Superdex 75 HR 10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA).

Molecular mass determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by fast protein liquid chromatography (FPLC)-gel filtration. SDS-PAGE was conducted following the protocol of Laemmli and Favre (48), using a 12% resolving gel and a 5% stacking gel. At the conclusion of electrophoresis, staining of the gel with Coomassie brilliant blue was carried out. FPLC-gel filtration was performed using a Superdex 75 HR 10/30 column that had been calibrated with molecular mass standards using an AKTA Purifier (GE Healthcare).

Analysis of N-terminal amino acid sequence. The amino-acid sequence of the purified protein was determined by means of automated Edman degradation. The amino acid sequence was determined using an HP G1000A Edman degradation unit and an HP 1000 HPLC system (Agilent Technologies, Santa Clara, CA, USA).

Assay for RNase activity. The activity of the purified RNase toward yeast tRNA (Sigma) was assayed by determining the generation of acid-soluble UV-absorbing species (reaction products) with the method of Wang and Ng (52). The RNase was incubated with 200 μg tRNA in 150 μg of 100 mM MES buffer (pH 6.0) at 37˚C for 1 h. The reaction was terminated by the addition of 350 μl of ice-cold 3.4% perchloric acid. After remaining on ice for 15 min, the sample was centrifuged (15,000 x g) at 4˚C for 15 min. The OD_{260} of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that induces an increase in OD_{260} of one per minute in the acid-soluble fraction per milliliter of reaction mixture under the specified conditions.

Activity of RNase toward polyhomoribonucleotides. The ribonucleolytic activity of the purified RNase toward various polyhomoribonucleotides as substrates was determined with a modification of a previously described method (33). The incubation of RNase with 100 μg poly(A), poly(C), poly(G) or poly(U) was carried out at 37˚C for 1 h in 250 μl of 100 mM sodium acetate buffer (pH 5.0), prior to the addition of 250 μl of ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate to terminate the reaction. The reaction mixture was left on ice for 15 min prior to centrifugation at 15,000 x g for 15 min at 4˚C. After appropriate dilution, the absorbance of the supernatant was read at 260 nm [for substrates poly(A), poly(G) and poly(U)] or at 280 nm [for substrate poly(C)].

Assay for ability to inhibit human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). The assay to determine the HIV-1 RT inhibitory activity of *Hohenbuehelia serotina* RNase was executed using a non-radioactive reverse transcriptase ELISA kit (Sigma-Aldrich, Trading Co., Ltd., Shanghai, China) as previously described (49).

Assay for anti-proliferative activity of RNase isolated from Hohenbuehelia serotina. The anti-proliferative activity of the purified RNase was determined as follows: the L1210 and MBL2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 mg/l streptomycin and 100 IU/ml penicillin, at 37˚C in a humidified atmosphere of 5% CO_2. Cells ($1x10^4$) in their exponential growth phase were seeded into each well of a 96-well culture plate and incubated for 3 h prior to the addition of the purified RNase followed by incubation for a further 48 h. The radioactive precursor, 1 μCi [^{3}H-methyl]-thymidine, was added to each well followed by incubation for 6 h before the cultures were harvested by means of a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting, as previously described (40).

Table I. Yields (from 650 g fresh *Hohenbuehelia serotina* fruiting bodies) and RNase activity (determined in 0.1 M MES buffer, pH 6.0, 37˚C) of various chromatographic fractions.

Fraction	Yield (mg)	RNase activity (U/mg)	Fraction	Yield (mg)	RNase activity (U/mg)
Extract	2018	35.7	CM1	19.0	<1
D1	459	98.6	CM2	13.4	51.8
D2	403	12.5	CM3	23.5	983.0
D3	529	<1	SU1	3.6	102.8
B1	165	<1	SU2	6.9	2705.3
B2	79.3	369.5	SU3	5.9	86.1
B3	81.2	15.7			

RNase, ribonuclease.

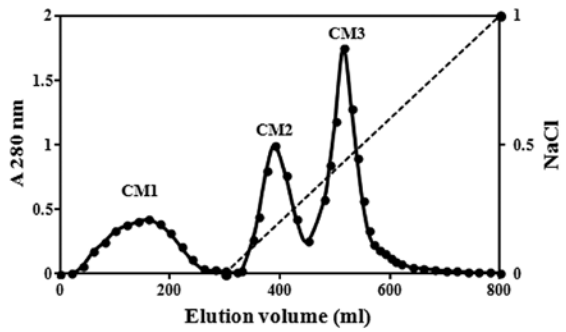

Figure 1. Ion exchange chromatography on CM-Sepharose. Sample: fraction B2 which was previously adsorbed on Affi-gel blue gel. Column dimensions: 2.5x20 cm. Starting buffer: 10 mM NH_4Ac (pH 5.2). Slanting dotted line across the right side of the chromatogram represents the linear 0-1 M NaCl gradient used to elute adsorbed proteins.

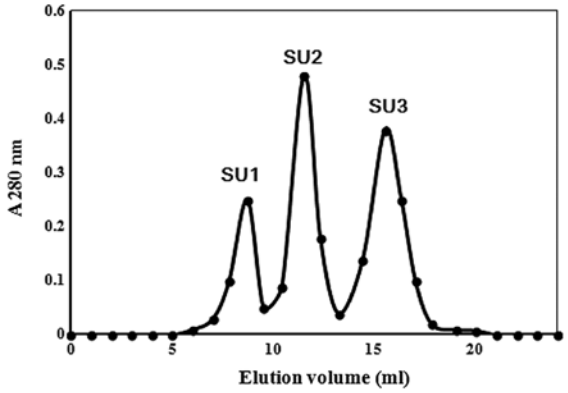

Figure 2. FPLC-gel filtration of fraction CM3 on a Superdex 75 HR10/30 column. Buffer, 0.2 M NH_4HCO_3 (pH 8.5); flow rate, 0.4 ml/min; fraction size, 0.8 ml.

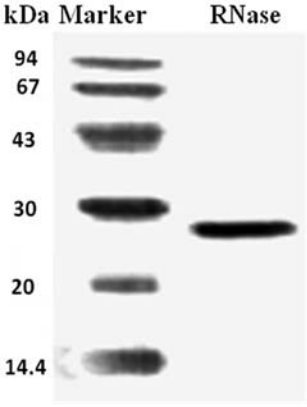

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified *Hohenbuehelia serotina* ribonuclease (RNase) (fraction SU2). The molecular mass of the marker proteins are, from top to bottom, 94, 67, 43, 30, 20 and 14.4 kDa.

Results

Isolation of RNase. Ion exchange chromatography of the extract on DEAE-cellulose yielded three fractions D1, D2 and D3 containing similar amounts of proteins. RNase activity was found only in the unadsorbed fraction D1. D1 was separated on Affi-gel blue gel into one unadsorbed and also the largest fraction (B1), together with two adsorbed fractions (B2 and B3). Fraction B2 was the only fraction with strong RNase activity. This fraction was resolved on CM-cellulose into an unadsorbed fraction CM1 devoid of RNase activity, an adsorbed fraction CM2 with weak RNase activity, and the most strongly adsorbed fraction CM3 containing the bulk of RNase activity (Fig. 1). Fraction CM3 was resolved into three fractions, SU1, SU2 and SU3 upon gel filtration on Superdex 75. RNase activity resided in the second fraction SU2 (Fig. 2 and Table I).

Determination of molecular mass. Fraction SU2 appeared as a single band with a molecular mass of 27 kDa, as shown by SDS-PAGE (Fig. 3).

Analysis of N-terminal amino acid sequence. The amino acid sequence was abtained by an HP 1000 HPLC system depending on Edman degradation. The N-terminal sequence was as follows: TVGGSLAEKGN, which showed homology to other fungal RNases to a certain degree (Table II).

Determination of optimum pH and optimum temperature. The optimum pH (Fig. 4) and temperature (Fig. 5) for the purified RNase were pH 5.0 and 80˚C, respectively.

Determination of polyhomoribonucleotide specificity. The RNase exerted a ribonucleolytic activity of 455.1, 311.2, 119.4 and 105 U/mg toward poly(C), poly(U), poly(A) and poly(G), respectively.

HIV-1 RT inhibitory activity. The RNase inhibited HIV-1 RT with an IC_{50} of 50 μM (Table III).

Anti-proliferative activity toward tumor cells. The RNase inhibited [^{3}H-methyl]-thymidine uptake by L1210 cells and MBL2 cells with an IC_{50} of 25 and 40 μM, respectively (Table III).

Table II. N-terminal sequence of *Hohenbuehelia serotina* (HS) ribonuclease (RNase) in comparison with RNases isolated from other mushrooms.

RNase	N-terminal sequence
HS:	TVGGSLAEKGN
TG:	DADIAVWAPPVNAQN
CM:	ETAHTHAGIQYSTVDVNNSIMKAVGGGAGN
PP:	AISANNERKGVNQQSVQNTYQENDV
VV:	APYVQLFRPLIQPQVLATFAIANNMAQY
LE:	ISSGCGTTGALSCSSNAKGTCCFEAPGGLI
IL:	VNSGCGTSGAESCSNSDDGTCCFEAPGGLL
DI:	GQPRQPQPQLLV
PE:	GEVVQYYP
PS:	DNGEAGRAAR
GL:	HLPBVPSFAYGSIKVYIN
RV:	TDHTLDTMMTHTLRD
PO:	ETGVRSCNCAGRSFTGTDVTNAIRSARAGGSGN
PT:	ALTAQDNRVRVGNRIVGNNFNFAAVQAAYY

TG, *Thelephora ganbajun*; CM, *Clitocybe maxima*; PP, *Pleurotus pulmonarius*; VV, *Volvariella volvacea*; LE, *Lentinus edodes*; IL, *Irpex lacteus*; DI, *Dictyophora indusiata*; PE, *Pleurotus eryngii*; PS, *Pleurotus sajor-caju*; GL, *Ganoderma lucidum*; RV, *Russulus virescens*; PO, *Pleurotus ostreatus*; PT, *Pleurotus tuber-regium*.

Table III. Inhibition rates (%) of RNase on growth of MBL2 cells, L1210 cells, and HIV-1 RT.

	Inhibition rates (%)		
Dose (μM)	HIV-1 RT	L1210	MBL2
10	13.5±0.9	24.6±1.8	18.7±1.1
20	21.4±1.6	40.9±2.7	31.4±2.3
40	42.1±2.7	72.1±5.2	49.7±3.8
80	73.4±4.3	94.3±5.5	73.6±5.9
IC_{50} (μM)	49.9	24.8	40.3

RNase, ribonuclease; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase.

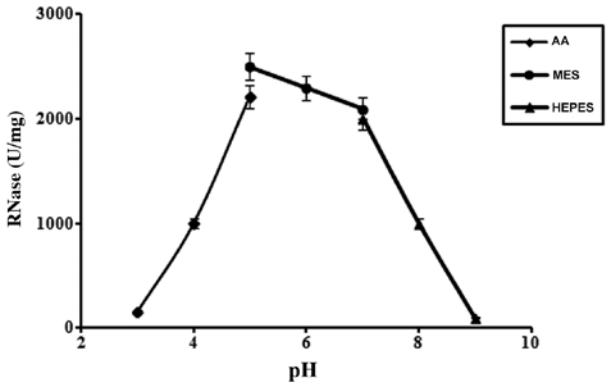

Figure 4. pH dependence of *Hohenbuehelia serotina* ribonuclease (RNase). Temperature used: 37˚C. Duration of incubation: 15 min. Buffer concentration: 0.1 M.

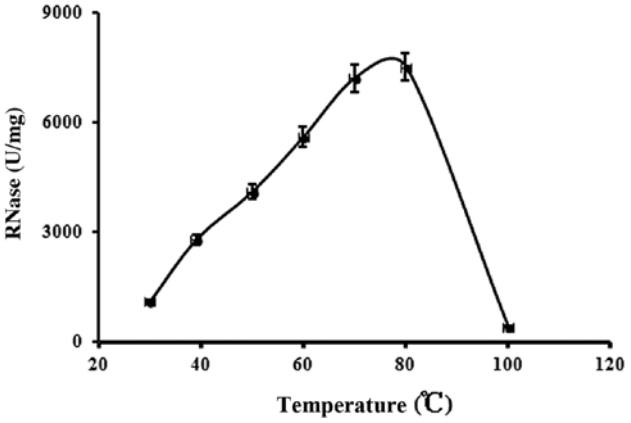

Figure 5. Temperature dependence of *Hohenbuehelia serotina* ribonuclease (RNase). Buffer used: 0.1 M NH_4Ac buffer (pH 4.5). Duration of incubation, 15 min.

Discussion

RNase isolated from *Hohenbuehelia serotina* is characterized by an N-terminal sequence distinctly different from that of previously reported mushroom and non-mushroom RNases. The molecular weight of previously reported mushroom RNases ranges from 9 to 42.5 kDa. The molecular mass of *Hohenbuehelia serotina* RNase (27 kDa) lies within this range. It is larger than the mass of RNases isolated from *Clitocybe maxima* (42), *Hypsizigus marmoreus* (45), *Lyophyllum shimeiji* (47), *Pleurotus djamor* (50), *Pleurotus eryngii* (51), *Pleurotus ostreatus* (31), *Pleurotus pulmonarius* (35), *Pleurotus sajor-caju* (40), *Russula delica* (46) and *Thelephora ganbajun* (43), but smaller than the mass of RNases isolated from *Boletus griseus* (44), *Dictyophora indusiata* (52), *Ganoderma lucidum* (43), *Pleurotus tuber-regium* (34), *Russulus virescens* (37) and *Volvariella volvacea* (33). Its mass is close to that of *Dictyophora indusiata* RNase (28 kDa) and *Russulus virescens* RNase (28 kDa). All these RNases are monomeric, and all of them are acid RNases.

RNases isolated from different mushrooms may have different optimum pH values and temperatures for RNase activity. The optimum pH of *Hohenbuehelia serotina* RNase (pH 5.0) is similar to that of *Hypsizigus marmoreus* RNase (pH 5.0) [Guan *et al* (45)] and *Russula delica* RNase (pH 5.0) [Zhao *et al* (46)]; however, *Hohenbuehelia serotina* RNase is much more thermostable. The optimum temperature for the purified *Hohenbuehelia serotina* RNase was found to be 80˚C, which is the highest optimum temperature for RNases purified from mushrooms.

Some RNases display antiviral activity. Zinc-finger antiviral protein inhibits HIV-1 infection by selectively targeting multiply spliced viral mRNAs for degradation [Zhu *et al* (53)]. *Rana catesbeiana* RNase inhibits Japanese encephalitis virus (JEV) replication and enhances apoptosis of JEV-infected BHK-21 cells.

Li *et al* (54) suggested that the targeted RNase is an alternative anti-hepatitis B virus (HBV) agent. The HIV-1 RT

inhibitory activity of *Hohenbuehelia serotina* RNase has been shown to be similar to that of RNases isolated from other mushrooms, such as *Lyophyllum shimeiji* (47) and *Thelephora ganbajun* (43). The mechanisms responsible for the inhibitory effects on HIV-1 RT may involve protein-protein interaction, as in the case of the inhibition of HIV-1 RT by the homologous protease (55) and the inhibition of HIV-1 protease by cathelicidin [Wong *et al* (56)].

Some RNases exert inhibitory effects on tumor cells. The binding of AS RNase and CM-AS RNase to leukaemic cells from patients with chronic lymphatic leukaemia has been demonstrated by indirect immunofluorescence, while no binding to normal leucocytes and leucocytes from patients with other hemoblastoses was observed (24). Bovine seminal RNase (BS-RNase) manifests specific cytotoxic effects on tumor cells, and non-malignant cells are not affected. In view of the finding that success was met only when BS-RNase was applied intratumorally, the properties of BS-RNase were improved by attachment to polylactic acid nanoparticles. The nanoparticle preparation and pure BS-RNase showed no difference when tested against leukemia (MOLT-4) and lymphoma (H9) cell lines sensitive and resistant to cytarabine *in vitro*. The aspermatogenic and anti-embryonal activities were augmented in the nanoparticle preparation of BS-RNase. It remains to be seen how well BS-RNase attached to polylactic acid nanoparticles performs as an antitumoral agent *in vivo* [Michaelis *et al* (57)]. The anticancer effect of the amphibian RNase Onconase as demonstrated experimentally and in clinical trials has been reported (58). Plant RNases have also been shown to exhibit antitumor effects in a number of studies (18,20,22).

Mushroom RNases have been shown to be effective against hepatoma and breast cancer cells (46). Zhao *et al* (16) noted that *Schizophyllum commune* RNase had no effect on the proliferation of leukemia and lymphoma cells. Only a few mushroom RNases have been shown to inhibit the growth of leukemia cells (36,40,59,60). We found that the anti-proliferative activities of *Hohenbuehelia serotina* RNase toward L1210 cells are not as remarkable as those of *Pleurotus sajor-caju* RNase (40), but more effective than those of *Hypsizigus marmoreus* RNase (45). To our knowledge, we also demonstrated for the first time that mushroom RNases exhibit anti-proliferative activity toward MBL2 cells.

Some plant RNases have antifungal activity (27,34,61) and have been classified as one family of pathogenesis-related proteins. It is interesting to note that *Hohenbuehelia serotina* RNase, similar to all previously reported mushroom RNases, is devoid of antifungal activity. However, its antiproliferative activity against cancer cells and its inhibitory activity toward HIV-I RT signify that it is a defense protein. Its RNase activity can be deployed against invaders.

In conlcusion, the RNase isolated from *Hohenbuehelia serotina* in the present study is a novel RNase, as evidenced by a novel N-terminal sequence and a high optimum pH. It manifests potent anti-proliferative activity toward cancer cells and inhibitory activity toward HIV-1 RT. These biological activities are potentially exploitable. In this regard it is noteworthy that not all previously reported mushroom RNases were assayed for or demonstrate anti-proliferative and HIV-1 RT inhibitory activities.

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